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Short communication

The effect of chlortetracycline on faecal microbial populations in growing swine

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ABSTRACT

The effect of antimicrobial use on the gastrointestinal microbiota of food animals is of increasing concern as bacteria accumulate resistance to multiple antimicrobials. Only a small fraction of the gastrointestinal microbiome is culturable, complicating characterisation of the swine gastrointestinal ecosystem. The objective of this study was to determine the effect of a growth promotion dose (50 g/ton) of chlortetracycline on the phylogenetic diversity of bacteria from swine faeces using a culture-independent method. Four freshly weaned pigs were provided a grower ration of primarily corn (63.7%) and soybean meal (25.2%) for 21 days; on Day 21 for 4 weeks the diet of two pigs was medicated with 50 g/ton chlortetracycline. Faecal material was collected from each pig on Days 0, 14, 23, 28, 35, 42 and 49 for 454-pyrosequencing of the 16S rRNA gene. UniFrac analysis of pyrosequencing data showed no significant difference in bacterial diversity based on diet and among pigs ($P > 0.05$) fed the low-level dose of chlortetracycline. The most abundant phyla in both treatment groups were Firmicutes, Bacteroidetes, Proteobacteria and Spirochaetes. Higher concentrations of chlortetracycline (e.g. 200 g/ton or 400 g/ton) may be required to observe a shift in the gastrointestinal flora in swine faeces compared with the low-level dose in this study. Studies of broader scope are needed to understand thoroughly how growth-promoting antimicrobials influence the gut microflora and benefit food animal growth efficiency.

1. Introduction

The complex commensal microbial consortium of the gastrointestinal tract is not well characterised, yet it is important in preventing the establishment of bacterial pathogens as well as in maintaining animal health and, ultimately, food safety [1]. Identification of the diversity and roles of bacterial populations and functional groups in the gastrointestinal compartments of healthy food-producing animals could ultimately enhance disease prevention for animals and humans.

Swine can be colonised by bacteria that are not pathogenic to the animal host but are pathogenic to humans and are often able to stably colonise the food animal's gastrointestinal tract [2]. These potential zoonotic pathogens may be excreted into the environment or contaminate carcasses via faeces at slaughter [1]. Antimicrobials are used in all stages of pork production in the USA to prevent disease and to improve performance [3]. Application of antimicrobials in feed can alter the composition of the gastrointestinal microbiota [4,5]. In some cases this is beneficial to the animal; alternatively, it may be detrimental if opportunistic pathogens are provided the opportunity to colonise the disrupted gastrointestinal tract.

In the nursery and in grower/finisher phases of swine production, chlortetracycline has the largest estimate of use [6]. Therefore, the present study examined the effect of a growth promotion dosage of chlortetracycline on the faecal microbiota of healthy growing swine using 16S rRNA gene pyrosequencing.

2. Materials and methods

2.1. Swine management

All procedures in this study were approved by the Agricultural Research Center Animal Care and Use Committee (ACUC protocol 2010002). Four freshly weaned Yorkshire/Duroc crossbred pigs (28 days old) were fed a commercial grower swine ration composed of (dry matter basis) ground corn 63.7%, soybean meal 25.2%, spray-dried porcine plasma 2%, select fish meal 4%, soybean oil 3%, dicalcium phosphate 1.4%, vitamin/trace mineral mix 0.4%, lysine–HCl 0.2% and DL-methionine 0.1%. The diet was formulated

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according to the US National Research Council's recommendations and pigs were provided access to water and minerals ad libitum. The US Food and Drug Administration (FDA)-approved usage levels for chlortetracycline to increase weight gain and to improve feed efficiency are 10–50 g/ton. The high boundary of 50 g/ton was used because it was hypothesised to be more likely to affect the normal flora in the swine intestinal tract.

2.2. Sample collection

All pigs were housed in the same barn for 49 days. On Day 21, two pigs were moved to an adjacent pen separated by cinder-block walls and were fed the previous diet medicated with 50 g/ton chlortetracycline (Alpharma, Bridgewater, NJ) (experimental group) from Days 21–49. Two pigs remained on the control diet for the duration of the study (control group). Faecal material was collected per rectum from each pig on Days 0, 14, 23, 28, 35, 42 and 49. Swine faecal samples were shipped overnight immediately after collection to the Research and Testing Laboratory (Lubbock, TX) for DNA extraction and pyrosequencing.

2.3. Massive parallel 16S rRNA gene pyrosequencing

Total genomic DNA was extracted from faecal samples using a QIAamp® DNA Stool Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. DNA samples were quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France). Bacterial tag-encoded FLX-titanium amplicon pyrosequencing (bTEFAP) based upon the V1–V3 region (*Escherichia coli* position 27–519) of the 16S rRNA gene was performed at the Research and Testing Laboratory as described previously, with the following primers: forward28F, GAGTTTGATCNTGGCTCAG; and reverse519R, GTNTTACNGCGGCKGCTG [7].

2.4. Sequence analysis

Raw sequence data were screened, trimmed and filtered with default settings using the QIIME pipeline v.1.4.0 (http://qiime.org) [8]. Chimeras were detected and excluded using the software B2C2 (http://www.researchandtesting.com/B2C2.html) [9]. Operational taxonomic units (OTUs) were defined as sequences with ≥97% similarity using QIIME. For classification of sequences on a genus level, the naïve Bayesian classifier within the Ribosomal Database Project (RDP) v.10.28 was used. The confidence threshold in RDP was set to 80%.

Alpha diversity (i.e. rarefaction) and beta diversity measures were calculated and plotted using QIIME. Differences in microbial communities between sample groups were investigated using the phylogeny-based unweighted UniFrac distance metric. To determine whether any sample group contained significantly different bacterial communities, the analysis of similarities (ANOSIM) function in the statistical software package PRIMER 6 (PRIMER-E Ltd., Luton, UK) was used on the unweighted UniFrac distance matrix [10].

3. Results

3.1. Taxonomic distribution of swine faecal microbiota

A total of 142 263 reads were generated with an average of 5080 ± 2070 reads per sample. To account for unequal sequencing depth across samples, subsequent analysis was performed on a randomly selected subset of 2000 sequences per sample. This number was chosen to avoid exclusion of samples with a lower number of sequence reads from further analysis. A total of 28 samples were taken both in the control and experimental groups. The most abundant phyla in both treatment groups were Firmicutes, Bacter-

Table 1
Most prevalent bacterial phyla (%) from each pig fed an unmedicated control diet.

Phylum	Pig	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Firmicutes	21	61.19	61.93	69.38	53.20	55.24	54.65	52.61
Bacteroidetes	21	24.31	29.33	13.97	23.09	18.76	20.90	28.16
Proteobacteria	21	13.00	4.58	11.88	18.31	3.17	10.98	8.43
Spirochaetes	21	0.77	2.87	3.78	4.54	21.81	12.76	10.41
Tenericutes	21	0.05	0.12	0.02	0.05	0.05	0.18	0.02
Chlamydiae	21	0.02	0.09	0.06	0.21	0.07	0.10	0.11
Firmicutes	30	61.34	59.29	86.89	48.84	54.78	75.14	58.96
Bacteroidetes	30	31.19	28.14	8.57	22.58	27.13	20.31	24.68
Proteobacteria	30	4.39	7.40	2.64	18.66	3.21	2.58	10.80
Spirochaetes	30	2.29	4.43	1.48	9.55	13.42	0.73	5.08
Tenericutes	30	0.13	0.05	0	0.02	0.18	0.12	0.05
Chlamydiae	30	0.09	0.02	0	0.02	0.06	0	0

Table 2
Most prevalent bacterial phyla (%) from each pig fed a chlortetracycline-medicated diet from weeks 4–7.

Phyla	Pig	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Firmicutes	19	64.60	72.21	69.85	51.79	55.54	53.77	68.57
Bacteroidetes	19	24.97	23.99	19.75	11.66	32.64	28.29	22.44
Proteobacteria	19	3.88	2.58	7.27	34.59	3.19	10.95	3.82
Spirochaetes	19	5.41	0.46	2.65	1.60	6.40	6.20	4.19
Tenericutes	19	0.05	0.01	0.10	0	0.27	0.04	0.19
Chlamydiae	19	0.28	0.16	0.08	0.02	0.23	0.06	0.04
Firmicutes	29	61.50	51.22	85.30	56.56	51.05	71.85	61.70
Bacteroidetes	29	32.53	33.57	5.28	20.82	17.50	18.77	27.40
Proteobacteria	29	4.03	6.68	5.31	10.16	3.75	4.89	5.18
Spirochaetes	29	0.56	7.52	3.67	11.63	26.67	3.47	4.91
Tenericutes	29	0.15	0.02	0.02	0.05	0.07	0	0.03
Chlamydiae	29	0.07	0.05	0	0.14	0.16	0.10	0.08

Chlortetracycline-medicated time points are shaded in grey.

oidetes, Proteobacteria and Spirochaetes. There was no statistically significant difference ($P > 0.05$) in the bacterial profiles based on diet or between individual pigs for phyla, families or genera. The predominant bacterial phylum, both for the unmedicated and chlortetracycline-medicated groups, was the Firmicutes, with a mean abundance of 63.18% and 60.33% for all 7 weeks, respectively. Bacteroidetes followed in abundance, with 23.10% and 23.02% for unmedicated and chlortetracycline-medicated groups, respectively (Tables 1 and 2). Fig. 1 compares the proportion of the six most abundant phyla from pigs fed the unmedicated control diet (Fig. 1a) and the chlortetracycline-medicated diet (Fig. 1b).

3.2. Rarefaction analysis of 16S rRNA gene sequences

The Chao1 and Ace richness estimators were used to estimate the total number of OTUs in the swine faeces. Rarefaction of the observed number of OTUs was saturated for samples based on diet and between individual pigs. No statistically significant difference ($P > 0.05$) in the OTUs was observed between the control group and the chlortetracycline-medicated group or between any of the individual pigs.

3.3. Microbial communities in controls and chlortetracycline-medicated pigs

Based on the unweighted UniFrac distance metric analysis of the 16S rRNA sequence data, there was no significant difference (ANOSIM; $P > 0.05$) in bacterial diversity between pigs fed the medicated or control diets, nor was there was a significant difference in bacterial diversity between the individual pigs (ANOSIM; $P > 0.05$).

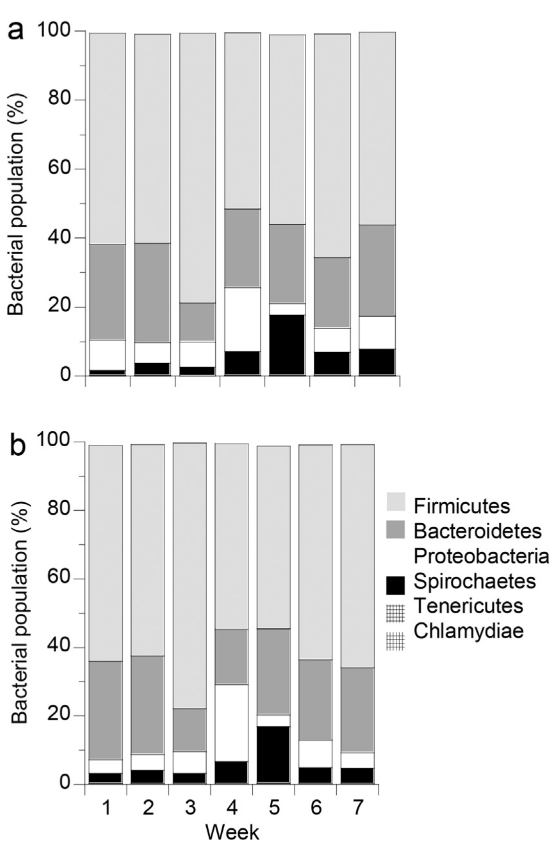

Fig. 1. Proportion of the bacterial population comprised of each phyla (for the six most abundant phyla) from pigs fed (a) control rations and (b) chlortetracycline (50 g/ton)-medicated rations from weeks 4–7.

4. Discussion

In recent years, a number of culture-independent molecular technologies have been developed that provide a more complete bacterial profile of the gastrointestinal microbiota [11–13]. Understanding variations in the microbial profile of healthy and sick animals, or antimicrobial-medicated and non-medicated animals, may make it possible to restore more appropriate microflora (via probiotics) to the animal's gastrointestinal tract, thus preventing colonisation by pathogens and multidrug-resistant (MDR) bacterial strains or shortening the length of disease symptoms without the need for antimicrobials.

When the microbial profiles were compared between swine fed a chlortetracycline-medicated or unmedicated diet, there was no statistically significant difference in the bacterial profiles based on diet or between individual pigs from phyla through genera. The predominant bacterial phylum, both for the unmedicated and chlortetracycline-medicated groups, was the Firmicutes, with a mean abundance of 63.18% and 60.33% for all 7 weeks, respectively. Bacteroidetes followed in abundance with 23.10% and 23.02% for unmedicated and chlortetracycline-medicated groups, respectively. This is in agreement with a metagenomic analysis of the swine gut microbiome that also showed Firmicutes and Bacteroidetes phyla to be the most prevalent [13,14].

Continuous feeding of the antimicrobial growth promoters tylosin and virginiamycin to commercial growing/finishing swine did not alter the microbial faecal community, suggesting that the action of growth promoters may occur in the gastrointestinal tract prior to reaching the colon [14]. The results of the study presented here may suggest a similar conclusion; however, caution should be taken when analysing these data since they represent a small cohort of pigs. A recent study [4] showing a shift in three microbial communities in the ileal contents of weanling piglets fed chlortetracycline may suggest that the effect of antimicrobial growth promoters are located in the ileum. However, the effects of growth-promoting antimicrobials may differ considerably among swine of different ages.

Since antimicrobial resistance profiles were not characterised for any bacterial constituents of the microflora, it is not known whether changes in the antimicrobial resistance profiles of the gastrointestinal microflora occurred due to treatment with chlortetracycline.

In a related contemporaneous study, differences in competitive fitness among *E. coli* strains with different plasmid profiles were examined when grown in in vitro fermentations with commensal faecal bacteria from the pigs in this study [15]. Five MDR *E. coli* strains that possessed none, two, six or eight plasmids were inoculated into anoxic faecal cultures from swine fed unmedicated or chlortetracycline-medicated diets. On Day 21 (42 days total) of chlortetracycline supplementation, faecal growth competition studies were performed. MDR *E. coli* were enumerated at 0, 6 and 24 h. The plasmid-free strain was below culturable limits both in the control and experimental cultures by 24 h. For each plasmid-bearing strain there was no statistically significant difference in population CFU/mL ($P > 0.05$) between the control and experimental cultures. This correlates with the results of this study, indicating that there was no significant effect on the faecal microflora due to the inclusion of chlortetracycline in the swine diets, therefore it would be unlikely that a difference in *E. coli* growth in the presence of faecal cultures would be detected between diets. Collectively, our results suggest that chlortetracycline alone at a low-level dose (50 g/ton) had little effect on the bacterial diversity or populations of swine faeces.

In a study that analysed the intestinal microbiome of swine after application of a performance-enhancing mixture of three antimicrobials (chlortetracycline 100 g/ton, sulfamethazine 100 g/ton and penicillin 50 g/ton; known as ASP250) [5], a shift in bacterial phylotypes occurred after 14 days. An increase in the *E. coli* population was observed as a component of this shift. The metagenomic analysis revealed an increase in genes related to energy production and conversion as well as antimicrobial resistance genes [5]. Importantly, the Lofft et al. study showed an increase in antimicrobial resistance genes not related to the antimicrobials used in the study. This suggests linkage of resistance genes on the same genetic element as those that confer resistance to the antimicrobials in ASP250. However, five antimicrobial resistance genes were present at high frequency both in medicated and unmedicated swine.

5. Conclusions

In the present study, feeding of low levels of chlortetracycline (50 g/ton) over a period of 4 weeks did not alter the microbial population or diversity in swine faeces. Although the use of chlortetracycline did not result in a detectable change in bacterial diversity, it was not determined whether there were changes in the antimicrobial resistance profile of the resident microflora.

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Competing interests

None declared.

Ethical approval

All procedures in this study were approved by the Agricultural Research Center Animal Care and Use Committee (ACUC protocol 2010002).

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